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PRINCIPAL INVESTIGATOR: Andrew Dennis
Bert O'Malley, M.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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13. ABSTRACT (Maximum 200 Words) We previously reported that human ubiquitin-activating enzyme 3 (hUba3), a novel candidate coactivator protein, had been identified by our lab in a yeast 2-hybrid screen using the PR-LBD in the presence of RU486 as a bait protein. Human Uba3, as well as its hetero-dimeric binding partner, APP-BP1, were able to enhance ligand-activated ER α , PR-B, GR, AR, TR β , and RAR α -dependent transcription in HeLa cells, although fold-activation was highest with ER α and PR-B. In a classic reverse-squelching experiment between ER α and PR-B, we demonstrated that both hUba3 and APP-BP1 exist in limiting quantities within the cellular milieu. Interestingly, hUba3 and APP-BP1 appear not to contain appreciable intrinsic activation functions nor demonstrate physical <i>in vitro</i> associations with PR-B, suggesting these proteins may differ from other previously identified coactivators. Furthermore, hUba3 indirectly interacts with PR-B in a progesterone-dependent manner in a mammalian one-hybrid system. Our observations suggest that hUba3 and APP-BP1 do not fit any classic coactivator models and may be unique in their own right. We are currently developing a chromatin immunoprecipitation assay system to gain further insight as to how these proteins regulate steroid receptor-dependent transcription of endogenous target genes.			
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Introduction

Two Steroids, estrogen and progesterone, regulate the growth and development of the mammary gland via a signaling cascade that is initiated through binding to their cognate nuclear hormone receptors (NHRs), the estrogen receptor (ER) and progesterone receptor (PR), members of the ligand-inducible transcription factor superfamily (1,2). Estrogen and progesterone receptor-targeted genes are further regulated by recruitment of coactivator proteins, which positively enhance transcription potential (3). To date many coactivator proteins have been described with separable or important enzymatic functions concomitant with their role as transcription coregulators (e.g. SRC-1, -2, -3, E6-AP, CARM-1, etc.) (4).

We previously described the identification of human ubiquitin-activating enzyme 3 (hUba3) as an RU486-bound PR-interacting protein in a yeast 2-hybrid system. Human Uba3 belongs to the neural-expressed developmentally down-regulated 8 (NEDD8) ubiquitin-like protein modification pathway that is distinct from the ubiquitin degradation pathway (5). The NEDD8 pathway functions through a pathway-specific E1 (a heterodimer of hUba3 and APP-BP1) and E2 enzyme (hUbc12), but lacks an E3 enzyme (6). The NEDD8 protein is primarily nuclear and the Cullin/ CDC53 family of proteins have been the only identified protein targets of this pathway (7). Interestingly, Cullin proteins have been shown to be part of an E3 multiprotein complex, termed the SFC complex (8). Recent evidence suggests that NEDD8 modification of Cullin proteins appears to control the ubiquitin ligase activity of the SCF complex and presumptive degradation of substrate proteins, such as p27^{Kip1} and I κ B α (9,10,11).

Our Initial studies of hUba3 demonstrated its ability to coactivate several steroid and non-steroid receptors in a transient-overexpression transcription system in HeLa cells. In agreement with Aim One in the Objectives, we wished to further characterize hUba3 as a coactivator of the NHR superfamily, namely ER α and PR-B. Since we also planned to evaluate the enzymatic function of hUba3, we extended our interests to other members of the NEDD8 pathway (APP-BP1 and hUbc12).

Body

Precise regulation of gene expression requires that transcription factors and associated proteins exist in limited intracellular quantities. This may be demonstrated in a classic reverse-squelching experiment whereby gradual addition of exogenous protein relieves squelching between ligand-activated ER α and PR-B. As shown in figure 1a and b, both Uba3 and APP-BP1 were able to reverse-squelching between ER α and PR-B, indicating that expression of these proteins are limited.

Coactivators also possess intrinsic activation functions that either serve as interaction sites for other transcription proteins or are indicative of an enzymatic activity involved in transcription, such as histone acetyltransferase (HAT) activity. We subcloned hUba3 and APP-BP1 into a Gal4DBD mammalian expression vector. Using a UAS_{Gal4} regulated reporter gene, we determined if Gal4DBD UBA3 and Gal4DBD APP-BP1 were able to activate the reporter gene, indicating presence of an intrinsic activation function. We also considered the possibility that coexpression of their heterodimeric counterpart may be necessary to observe this activity. In both scenarios hUba3 and APP-BP1 did not appear to contain appreciable activation functions (figure

2a and b), suggesting uniqueness from other previously characterized coactivators.

Most coactivators have been shown to interact directly with nuclear hormone receptors in an agonist-dependent manner *in vitro*. We performed *in vitro* GST pulldown experiments with ³⁵S-labeled hUba3 or APP-BP1 and GSTPR-B in the presence of vehicle control, progesterone, or RU486, to determine hormone-dependent interactions between these proteins. Our results demonstrate that hUba3 and APP-BP1 do not physically interact with PR-B in any of the hormone conditions tested (figure 3a and b). As a positive control we were able to show that hUba3 and GSTAPP-BP1 were able to associate with each other (figure 3c). On the basis that interactions may occur through additional proteins not present *in vitro*, we resorted to mammalian one-hybrid interactions using PR-B and VP16Uba3 in HeLa cells. Interestingly, both the c-terminal and n-terminal halves of Uba3 were able to interact in a hormone-dependent manner compared to the empty vector VP16 control (figure 3d). These results suggest that hUba3 and PR do not physically interact, but likely interact through other proteins involved in transcription.

Key Research Accomplishments

- Established that both hUba3 and APP-BP1 can reverse squelching between ligand-activated ER α and PR-B, demonstrating that both proteins exist in limiting intracellular quantities.
- Human Uba3 and APP-BP1 do not appear to possess intrinsic activation functions, but Western blotting is still needed to confirm protein expression.
- Analysis of direct physical *in vitro* interactions between GSTPR-B (- hormone, + progesterone, and + mifepristone) and hUba3 or APP-BP1 demonstrated that these proteins do not physically interact.
- Mammalian one-hybrid interactions between PR-B and VP16Uba3 constructs demonstrated that both halves of hUba3 could be recruited to PR-B in a hormone-dependent manner.

Reportable Outcomes

The ongoing work described here was presented as a poster and an abstract at the Annual Endocrine Society Meeting (1999), held in Toronto, Canada.

Conclusions

Our evidence suggests that the coactivators, hUba3 and APP-BP1, show distinct coactivator behavior compared to other previously characterized coactivators (e.g. SRC-1, -2, -3, E6-AP, CARM-1, etc.) (4). These differences include in the ability to interact directly with PR-B in a hormone-dependent manner *in vitro* and lack of an intrinsic activation function. However, hUba3 and APP-BP1 were able to coactivate several members of the NHR superfamily (ER α , PR-B, AR, GR, TR β , and RAR α), and reverse-squelching between ligand-activated ER α and PR-B. Furthermore, the N-terminal and C-terminal halves of hUba3 were able to indirectly interact

with PR-B in a progesterone-dependent manner. From these results we conclude that hUba3 and APP-BP1 do not fit any classic coactivator model and may be unique in their own right. Our results have not determined the exact mechanism by which hUba3 and APP-BP1 act as coactivators in NHR-dependent transcription and additional experiments are needed to address their precise involvement in agreement with Aim One of the Objectives.

Recent evidence suggests that the NEDD8 pathway appears to play an important role in the regulation of the SCF ubiquitin ligase activity toward substrate proteins (8,9,10,11). Since recent studies with ER α suggest that its down-regulation is intimately tied to the process of transcription, it is possible that the NEDD8 pathway proteins and the SCF complex are part of this process (12,13). This would result in a transient association with transcription proteins during gene activation. This possibility is also in agreement with the results observed during this study. Interestingly, chromatin immunoprecipitation (ChIP) assays have recently been employed to describe the dynamics of ER α and coactivator protein assembly/ disassembly on estrogen-regulated endogenous promoters (such as cathepsin D) (14,15). Since hUba3 and APP-BP1 have been shown to coactivate ER α , this assay system allows us to address the dynamics of these proteins during ER α / coactivator assembly and disassembly. These experiments will provide greater insight to the involvement of hUba3 and APP-BP1 in NHR-dependent transactivation, in agreement with Aim One of the Objectives.

Appendices:

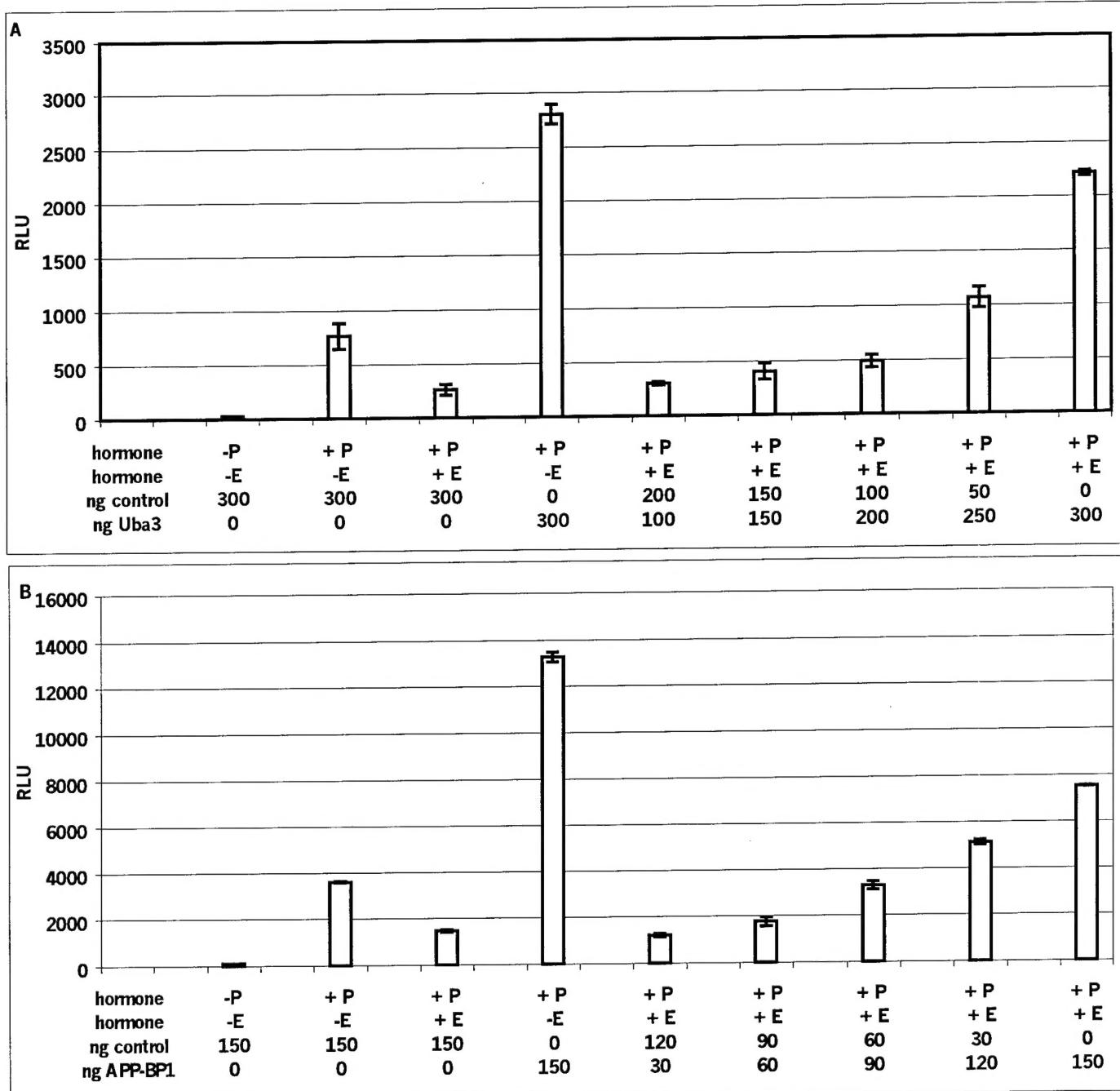


Figure 1. Human Uba3 and APP-BP1 reverse-squelching between ligand-activated ER α and PR-B in HeLa cells. Cells were transfected with 8ng pcDNA3.1 hPR-B, 16ng pcDNA3.1 hER α , 500ng PRE2 TATA Luciferase, and a balanced amount of control and coactivator DNA indicated in the chart above. A. Reverse-squelching experiment performed with hUba3. B. Reverse-squelching experiment performed with APP-BP1. Cells were harvested following 24 hour incubation and assayed for luciferase activity. The data are representative of three independent experiments and error bars depict three data points. The concentration of progesterone (P) and β -estradiol (E) were 10^{-8} M. RLU (Relative Light Units).

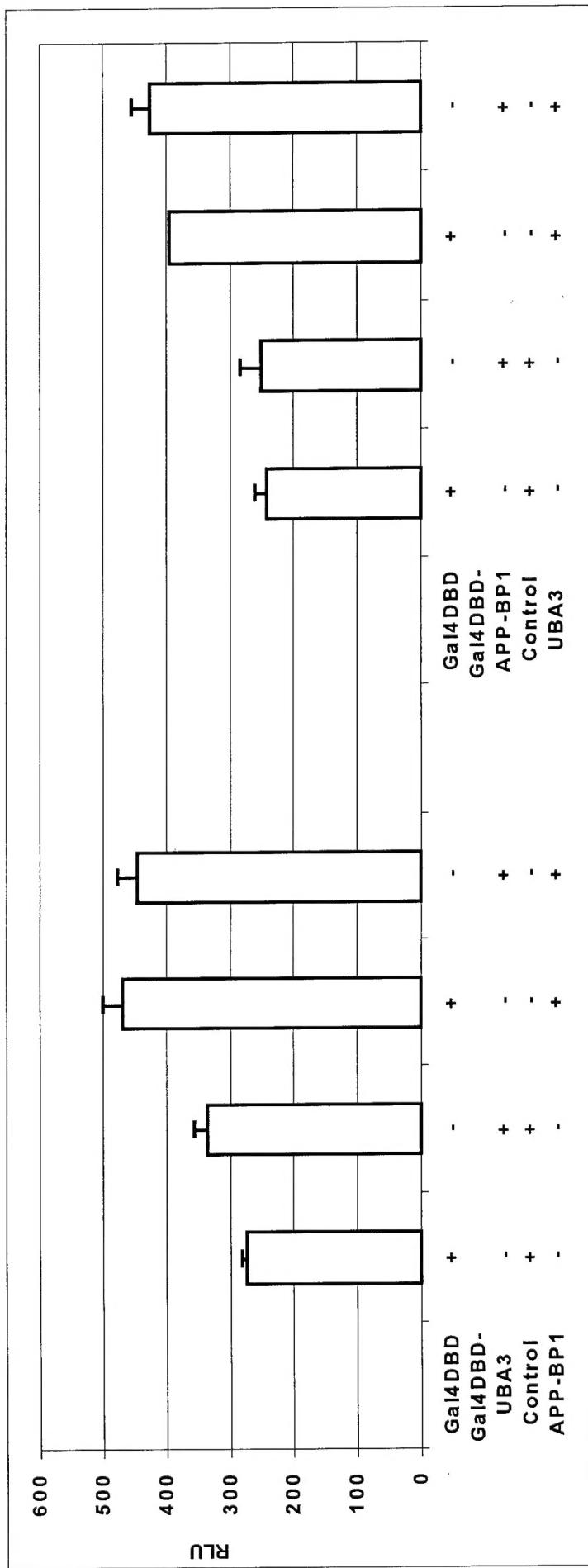


Figure 2. Gal4DBD UBA3 and GAL4DBD APP-BP1 do not possess intrinsic activation function in HeLa cells. Left: 200ng pBIND (Gal4DBD) or 200ng pBIND UBA3, and 200ng of control or APP-BP1, were transfected with 500ng pGS (UAS_{Gal4X4} Luciferase) reporter. Right: 200ng pBIND (Gal4DBD) or 200ng pBIND APP-BP1, and 200ng of control or hUBA3, were transfected with 500ng pGS (UAS_{Gal4X4} Luciferase) reporter. Cells were harvested following 24 hour incubation and assayed for luciferase activity. The data are representative of three independent experiments and error bars depict three data points. RLU (Relative Light Units).

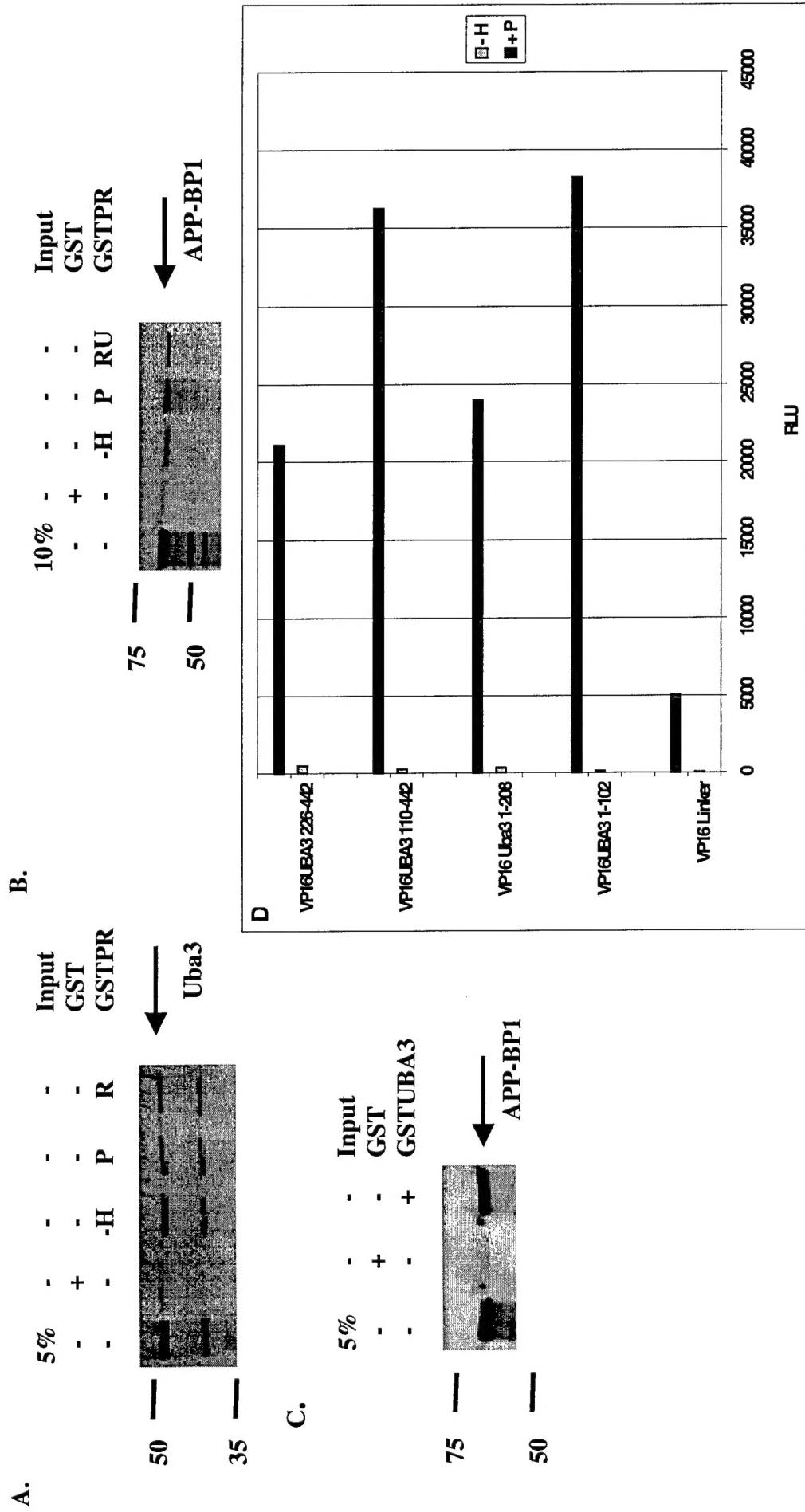


Figure 3. Human Uba3 and APP-BP1 do not physically interact *in vitro*, but interact indirectly with PR-B in HeLa cells. Baculovirus-expressed GSTPR was produced in Sf9 insect cells in the presence of ethanol control (-H), progesterone (P), or RU486 (R). GSTPR was loaded onto glutathione sepharose-A beads and assayed for interaction with *in vitro* transcribed/translated 35 S-labeled methionine. A. Interaction between GSTPR and 35 S-labeled hUba3. B. Interaction between GSTUBA3 and 35 S-labeled APP-BP1. C. GSTUBA3 was produced in *E. coli* and loaded onto glutathione sepharose-A beads. Interaction between GSTUBA3 and 35 S-labeled APP-BP1. D. Interaction domains between Uba3 and PR were determined by mammalian one-hybrid system with VP16AD-Uba3 fusion proteins and hPR expressed in HeLa cells. The concentration of hormone used was 10^{-8} M.

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